Selective progesterone receptor modulator asoprisnil induces endoplasmic reticulum stress in cultured human uterine leiomyoma cells

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Xu Q, Ohara N, Liu J, Nakabayashi K, DeManno D, Chwalisz K, Yoshida S, Maruo T. Selective progesterone receptor modulator asoprisnil induces endoplasmic reticulum (ER) stress-induced apoptosis in cultured human uterine leiomyoma cells. Am J Physiol Endocrinol Metab 293: E1002–E1011, 2007. First published July 24, 2007; doi:10.1152/ajpendo.00210.2007.—A recent clinical trial (Chwalisz K, Larsen L, Mattia-Goldberg C, Edmonds A, Elger W, Winkel CA. Fertil Steril 87: 1399–1412, 2007) has demonstrated that the selective progesterone receptor modulator asoprisnil efficiently causes the shrinkage of uterine leiomyoma. The present study was conducted to examine whether asoprisnil elicits endoplasmic reticulum (ER) stress-induced apoptosis in cultured human uterine leiomyoma cells. After subculture in phenol red-free DMEM supplemented with 10% FBS for 120 h, cultured cells were stepped down to serum-free conditions with or without graded concentrations of asoprisnil. ER stress-associated and apoptosis-related proteins were assessed by reverse transcription-PCR analysis or Western blot analysis. RNA interference of growth-arrest- and DNA-damage-inducible gene 153 (GADD153) was performed using small interfering RNA. Terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL)-positive rates were assessed by TUNEL assay. Compared with untreated control cultures, treatment with 10−7 M asoprisnil significantly (P < 0.05) increased the protein contents of ubiquitin at 2 h and phospho-double-stranded RNA-activated protein kinase-like ER kinase, phosphorylated (p)-eukaryotic initiation factor 2 (p-eIF2α), activating transcription factor 4, and glucose-regulated protein 78 kDa at 4 h, followed by the significant (P < 0.05) increase in GADD153 protein content at 6 h and cleaved poly(adenosine 5′-diphosphate ribose)polymerase (c-PARP) at 8 h. RNA interference of GADD153 suppressed protein contents of asoprisnil-induced cleaved PARP, Bak, Bak, GADD34, and tribbles-related protein 3 (TRB3) and TUNEL-positive rate but attenuated asoprisnil-induced reduction in Bcl-2 protein content in cultured leiomyoma cells. These results suggest that asoprisnil elicits ER stress-induced apoptosis in cultured leiomyoma cells and that GADD153 plays a role in asoprisnil-induced apoptosis by modulating the Bcl-2 family of proteins, GADD34, and TRB3.

SELECTIVE PROGESTERONE receptor modulators (SPRMs) represent a class of progesterone receptor (PR) ligands that exert tissue-selective P4 agonist, antagonist, or mixed agonist/antagonist effects on P4-target tissues (7). Asoprisnil belongs to the novel class of 11β-benzyldoxime-substituted SPRMs and has been studied as a potential new medical treatment of symptomatic uterine leiomyomata (9). Recent clinical studies with asoprisnil in patients with leiomyomata demonstrated a significant reduction in leiomyoma volume as well as an improvement in menorrhagia and pressure-related symptoms associated with leiomyomata (8). We (5, 36, 43) have recently demonstrated that asoprisnil not only inhibits cell proliferation of cultured leiomyoma cells but also induces apoptosis of these cells by activating tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptotic signaling and by down-regulating X-linked chromosome-linked inhibitor of apoptosis protein and Bcl-2 expression in the absence of comparable effects on myometrial cells, suggesting a cell-type specific PR antagonistic action of asoprisnil. However, whether endoplasmic reticulum (ER) stress-induced apoptosis is involved in asoprisnil-induced apoptosis of cultured leiomyoma cells remains to be clarified.

The ER is the site of synthesis and folding of secreted, membrane-bound, and organelle-targeted proteins (39). The conditions that interfere with ER function are referred to as ER stress (39), including perturbations in Ca homeostasis or redox status, elevated secretory protein synthesis, expression of misfolded proteins, and glucose deprivation (49). The ER triggers the unfolded protein response (UPR) to cope with accumulated unfolded or misfolded proteins (49). However, if ER stress is prolonged, apoptotic cell death ensues (40). The UPR is a prosurvival response mediated by ER chaperone protein Ig heavy chain-binding protein (BiP)/glucose-regulated protein 78 (GRP78) to prevent misfolded protein accumulation (45) and by three kinds of transmembrane ER signaling proteins: double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) (40, 45). On accumulation of unfolded proteins in the ER lumen, GRP78 disassociates from PERK, ATF6, and IRE1, allowing their activation. On removal of GRP78, PERK oligomerizes in ER membranes and undergoes autophosphorylation and then phosphorylates eukaryotic initiation factor 2α (eIF2α), thereby blocking general protein synthesis (40, 45). The phospho-eIF2α (p-eIF2α) induces the translation of ATF4 mRNA.

UTERINE LEIOMYOMAS are steroid hormone-dependent benign neoplasms originating from uterine smooth muscle cells. Traditionally, estrogen has been attributed to the pathogenesis of leiomyomata, but accumulating data provide novel evidence that progesterone (P4) also plays an important role in regulating the growth of uterine leiomyomata (22).

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ATF6 and IRE1 can induce ER chaperone proteins such as GRP78 and GRP94 (17). IRE1 is a type I transmembrane serine/threonine protein kinase with endoribonuclease activity. On triggering by the ER stress, activated IRE1 cleaves X-binding protein-1 (XBP-1) mRNA, and then the spliced form of XBP-1 induces ER chaperone GRP78 (17). On the other hand, PERK, ATF6, and IRE activate proapoptotic molecules such as growth-arrest- and DNA-damage-inducible gene 153/CCAAT/enhancer-binding protein b (GADD153/CHOP) (14, 40) during prolonged ER stress. GADD153 is shown to be involved in ER stress-induced apoptosis, as evidenced by the observations that overexpression of GADD153 promotes cell-cycle arrest and/or apoptosis but that a deficiency of GADD153 protects cells from ER stress-induced apoptosis (32). GADD153 targets several proapoptotic molecules, including GADD34 (21) and tribbles-related protein 3 (TRB3) (29). GADD34 overexpression was shown to enhance apoptosis of the cells treated with ionizing radiation (1) and causes the dephosphorylation of eIF2α (25). TRB3 is reported to be involved in ER stress-induced apoptosis in a GADD153-dependent manner (29).

On the other hand, the Bcl-2 family of proteins is known to play essential roles in apoptosis. They are divided into three groups based on the structure and role in apoptosis: 1) antiapoptotic proteins such as Bcl-2 and Bcl-XL; 2) proapoptotic proteins such as Bax and Bak; and 3) BH3-only proteins, including Bid, Bad, Noxa, and Puma (11). Recent studies have demonstrated that Bcl-2, Bax, and Bak are localized to the mitochondria as well as the ER (2, 51), suggesting their participation in the regulation of apoptotic crosstalk between the ER and mitochondria. Emerging evidence indicates that the intrinsic and extrinsic apoptotic pathways are closely linked with ER stress-induced apoptosis (40).

The present study was conducted to investigate the effects of graded concentrations of asoprisnil on the induction of ubiquitin protein content and the UPR signaling markers in cultured leiomyoma cells. For comparative purposes, cultured leiomyoma cells were treated with tunicamycin (ER stress inducer; an inhibitor of N-linked glycosylation) in some experiments. In addition, to address the role of GADD153 in asoprisnil-induced apoptosis of cultured leiomyoma cells, the effects of the silencing of GADD153 gene on the induction of cleaved poly(adenosine 5′-diphosphate-ribose) polymerase (PARP) and terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL)-positive rate as the indexes of programmed cell death, and the expression of Bcl-2 family of proteins were examined in the cells.

MATERIALS AND METHODS

Tissue collection. Twenty-seven samples of uterine leiomyoma tissues were obtained from Japanese women with regular menstrual cycles who underwent hysterectomy at Kobe University Hospital. Informed consent was obtained from each patient before surgery for the use of uterine leiomyoma tissues for the present study. The Institutional Review Board approved the use of uterine leiomyoma tissues for culture experiments. The patients ranged in age from 30–45 yr, with a mean age of 38.2 yr, and had received no hormonal therapy for at least 6 mo before surgery. The histological diagnosis of each uterine specimen was examined. Samples were excluded from the study if accurate menstrual cycle dates could not be assigned or if unexpected pathology was found (e.g., adenomyosis). Each uterine specimen was examined by a pathologist for histological evaluation. Endometrial tissues were obtained from the extirpated uterus, and the day of the menstrual cycle was determined by endometrial histological dating according to the method of Noyes et al. (26). Fifteen samples were collected from the proliferative phase of the menstrual cycle and twelve samples were from the secretory phase of the menstrual cycle.

Cell culture. Uterine leiomyoma tissues were obtained in the proliferative phase or secretory phase of the menstrual cycle. The central parts of leiomyoma tissues were collected with a careful removal of pseudocapules and fibrous septa materials. Tissues obtained were dissected from endometrial layers, cut into small pieces, and digested in 0.2% collagenase (wt/vol) at 37°C for 3–5 h (23). The collagenase treatment is shown to provide a pure population with smooth muscle cell characteristics without stromal or glandular epithelial cell contamination (23). The leiomyoma cells were collected by centrifugation at 460 g for 5 min and washed three times with PBS containing 1% antibiotic solution. The cell viability was determined by trypan blue exclusion test. The isolated leiomyoma cells were plated at densities of approximately 1 × 10⁴ cells/dish in 10-cm² culture dishes, 1 × 10⁵ cells/well in 24-well tissue culture plates, and 2 × 10⁴ cells/well in two-well chamber glass slides.

The isolated leiomyoma cells in culture dishes were subcultured at 37°C for 120 h in a humidified atmosphere of 5% CO₂–95% air in phenol red-free DMEM supplemented with 10% FBS (vol/vol; Invitrogen Life Technologies, Grand Island, NY). Since the proliferating cell nuclear antigen (PCNA)-positive rate of leiomyoma cells was shown to be higher in the secretory phase than in the proliferative phase of the menstrual cycle (38), we subcultured isolated cells in phenol red-free DMEM supplemented with 10% FBS for 120 h to abrogate the menstrual cycle-dependent influence on the biological characteristics of cells, confirming that the 120-h subculture produced no differences in the PCNA-positive rate of cultured leiomyoma cells obtained from the different phases (38). The monolayer cultures reaching ~70% confluence were treated with graded concentrations (10⁻⁸ M, 10⁻⁷ M, and 10⁻⁶ M) of asoprisnil (J867) (TAP Pharmaceutical Products, Lake Forest, IL) or 5 μg/ml tunicamycin (an inhibitor of N-glycosylation) (Sigma), in serum-free, phenol red-free DMEM. Asoprisnil was dissolved in absolute ethanol. The final concentration of ethanol in culture media was <0.01%, and the same concentration of ethanol was used as a vehicle in control cultures.

Western blot analysis. Proteins were extracted from cultured leiomyoma cells as described previously (46). The cells were lysed at 4°C for 20 min using a lysis buffer consisting of 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, 1 mg/l aprotinin, 0.1% SDS, and 50 mM Tris·HCl, pH 7.5. Whole cell lysates were subsequently centrifuged at 13,000 g for 30 min at 4°C, and the supernatants were collected. Protein content in the supernatants was determined by the Bradford assay. Each 100-μg aliquot of the proteins extracted from cells was electrophoresed on a 10% SDS-PAGE under reducing conditions. The proteins were electrophoretically transferred from gels to nitrocellulose membranes (BioRad Laboratories, Hercules, CA). The blots were exposed overnight to primary antibodies, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL). The antigen-antibody complexes were detected with the ECL chemiluminescence detection system (Amersham Biosciences). Membranes were visualized by exposure to X-OMAT film (Eastman Kodak, Rochester, NY). The radioautograms were scanned and quantified with ChemiImager 4400 (Astec, Osaka, Japan). The experiments were repeated with at least three different cultured specimens in triplicate with similar results, and the reported results are representative. The relative values of each protein were normalized with β-actin values from the same samples.

The following primary antibodies were used in this study: ubiquitin (sc-8017), p-PERK (sc-32577-R), AT4 (sc-7583), IRE1α (sc-10510), XBP-1 (sc-7160), GRP78 (sc-1051), GRP94 (sc-1794), GADD153 (sc-
RT-PCR analysis. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Chatsworth, CA). First-strand cDNA was synthesized from 2 μg total RNA using an Omniscript RT Kit (Qiagen). The human-specific PCR primers of GADD153 were synthesized by Nippon EGT Oligo (Toyama, Japan). The following sense and antisense primers were used to amplify human GADD153 (sense 5′-GGCAGCTGAGTCATTGCC-3′ and antisense 5′-GGCACGTACCGTCCGCTTCA-3′) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense 5′-GGGAAGCTCAGGGATGCGTCC-3′ and antisense 5′-CATGTTGGGCATGAGTCACCA-3′). PCR reactions were performed using the following parameters on a GeneAmp PCR System 9600-R (Perkin-Elmer, Norwalk, CT): 1 cycle of 50°C for 30 min, 95°C for 15 min, and then 35 cycles (GADD153) or 25 cycles (GAPDH) of 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, followed by a final extension step of 72°C for 5 min. PCR products were electrophoresed on a 1.5% agarose gel, and the bands were quantified with NIH Image version 1.60. The relative values of GADD153 mRNA were normalized with GAPDH mRNA values form the same samples. The experiments were repeated with at least three different cultured specimens in triplicate with similar results, and the reported results are representative.

In situ TUNEL assay. In situ labeling of fragmented DNA in cultured leiomyoma cells was performed with the TUNEL assay to detect apoptotic cell death using the ApopTag in situ apoptosis detection kit (Intergen, Purchase, NY) according to the manufacturer’s protocol for monolayer cultures. Leiomyoma cells were subcultured in two-well glass chamber slides and then cultured for the varying times in the absence or presence of graded concentrations of asoprisnil. At the termination of cultures, nucleotide-sized DNA fragments were tailed with digoxigenin-deoxy-UTP and then bound with peroxidase-conjugated anti-digoxigenin antibodies. The nuclei were counterstained with hematoxylin (Zymbed Laboratories, San Francisco, CA) for determining the TUNEL-positive rate of cultured leiomyoma cells form 24 h in accordance with the upregulation of cleaved PARP protein content (5). To elucidate an association of the progression of apoptotic cell death program with ER stress, we examined when cleaved PARP protein content is induced in cultured leiomyoma cells treated with 10⁻⁷ M asoprisnil as early as 4 h and remained elevated at 10 h (Fig. 1, A and B). We also examined IRE1α and XBP-1 protein contents as another branch of the UPR. However, asoprisnil treatment did not affect IRE1α and XBP-1 protein contents in cultured leiomyoma cells during the period of the experiment (Fig. 1, C).

Proapoptotic transcription factor GADD153 protein content was significantly (P < 0.05) increased in cultured leiomyoma cells treated with 10⁻⁷ M asoprisnil at 6 h and steadily rose up to 10 h (Fig. 1, A and B). PARP is the best-known caspase substrate, and its cleavage is universally adopted as an apoptotic hallmark (6). We previously demonstrated that 10⁻⁷ M asoprisnil increased TUNEL-positive rate of cultured leiomyoma cells from 24 h in accordance with the upregulation of cleaved PARP protein content (5). To elucidate an association of the progression of apoptotic cell death program with ER stress, we examined when cleaved PARP protein content is induced in cultured leiomyoma cells treated with 10⁻⁷ M asoprisnil. Cleaved PARP protein content was started to significantly increase (P < 0.05) at 8 h and still remained elevated at 10 h in the cells (Fig. 1B), indicating that asoprisnil induced the progression of the apoptotic machinery in cultured leiomyoma cells.

Effects of graded concentrations of asoprisnil and tunicamycin on the induction of ubiquitin protein content and the activation of the PERK-eIF2α-ATF4 UPR signaling pathway in cultured leiomyoma cells. The effects of graded concentrations of asoprisnil and 5 μg/ml tunicamycin on ubiquitin, p-PERK, p-eIF2α, and ATF4 protein contents in leiomyoma cells cultured for 4 h were assessed by Western blot analysis (Fig. 2). One-way ANOVA of the indexes for ubiquitin, p-PERK, p-eIF2α, and ATF4 protein contents showed significant effects of asoprisnil concentration (P < 0.001). Asoprisnil treatment at concentrations greater than or equal to 10⁻⁸ M significantly (P < 0.05) increased ubiquitin protein content in cultured leiomyoma cells, and tunicamycin treatment also increased ubiquitin protein content in the cells (Fig. 2A). Treatment with asoprisnil at concentrations greater than or equal to 10⁻⁸ M and 5 μg/ml tunicamycin significantly (P < 0.05) increased p-PERK protein content compared with untreated control cultures (Fig. 2B). Compared with untreated control cultures, p-eIF2α protein content was significantly (P < 0.05) increased in the cells treated with asoprisnil at concentrations greater than or equal to 10⁻⁷ M and 5 μg/ml tunicamycin (Fig. 2C).
Treatment with asoprisnil at concentrations greater than or equal to $10^{-7}$ M and 5 µg/ml tunicamycin significantly ($P < 0.05$) increased ATF4 protein content in cultured leiomyoma cells compared with untreated control cultures (Fig. 2D).

Effects of graded concentrations of asoprisnil and tunicamycin on the UPR downstream molecules in cultured leiomyoma cells. To examine the effects of asoprisnil on the UPR downstream molecules, GRP78, GRP94, and GADD153 protein contents were evaluated in leiomyoma cells cultured for 6 h by Western blot analysis (Fig. 3). One-way ANOVA of the indexes for GRP78, GRP94, and GADD153 protein contents showed significant effects of asoprisnil concentration ($P < 0.001$). Compared with untreated control cultures, GRP78 protein content was significantly ($P < 0.05$) increased in cultured leiomyoma cells treated with asoprisnil at concentrations greater than or equal to $10^{-7}$ M and 5 µg/ml tunicamycin (Fig. 3A), whereas GRP94 protein content was significantly ($P < 0.05$) increased in these cells treated with asoprisnil at concentrations greater than or equal to $10^{-7}$ M and 5 µg/ml tunicamycin (Fig. 3B). GADD153 protein content was significantly ($P < 0.05$) increased in cultured leiomyoma cells treated with asoprisnil at concentrations greater than or equal to $10^{-8}$ M and 5 µg/ml tunicamycin (Fig. 3C).
Effects of GADD153 RNA interference on asoprisnil-induced apoptosis of cultured leiomyoma cells. To explore the role of GADD153 in asoprisnil-induced apoptosis in cultured leiomyoma cells, we used the siRNA technique to knockdown GADD153 gene. The RT-PCR analysis revealed that the transfection of cultured leiomyoma cells with siRNA GADD153 (siGADD153) significantly \( (P < 0.01) \) suppressed asoprisnil-induced increase in GADD153 mRNA levels compared with nonspecific siRNA control (siControl) cells (Fig. 4A). In the absence of asoprisnil, GADD153 protein content was significantly \( (P < 0.05) \) decreased in the cells transfected with siGADD153 compared with siControl cells with the inhibition rate being \( \sim 60\% \) (Fig. 4B).

We examined whether the PERK-eIF2\(\alpha\)-ATF4 branch of the UPR is still activated by asoprisnil treatment both in siControl and siGADD153 cells. Western blot analysis revealed that p-PERK, p-eIF2\(\alpha\), and ATF4 protein contents were induced in cultured leiomyoma cells treated with \( 10^{-7}\) M asoprisnil in both siControl and siGADD153 cells and that the degree of response of the PERK, eIF2\(\alpha\), and ATF4 protein levels in both cells treated with asoprisnil was similar (Fig. 4C). This indicates that the effects of asoprisnil treatment on siGADD153 cells are due to GADD153 depletion, not by reducing ER stress per se or some property of the siRNA.

Cleaved PARP protein content was significantly \( (P < 0.05) \) increased in siControl and siGADD153 cells treated with \( 10^{-7}\) M asoprisnil compared with untreated controls (Fig. 4D). However, RNA interference of GADD153 resulted in a significant \( (P < 0.01) \) inhibition of asoprisnil-induced increase in cleaved PARP protein content compared with siControl cells treated with asoprisnil (Fig. 4D).

To test the appearance of apoptotic cultured leiomyoma cells in response to asoprisnil treatment, TUNEL assay was performed in siControl and siGADD153 cells cultured for 36 h in the presence or absence of \( 10^{-7}\) M asoprisnil. TUNEL-positive rate was significantly \( (P < 0.05) \) increased in siControl and siGADD153 cells treated with \( 10^{-7}\) M asoprisnil compared with untreated controls, respectively (Fig. 4E). However, RNA interference of GADD153 caused a significant \( (P < 0.01) \) decrease in asoprisnil induction of TUNEL-positive rate of cultured leiomyoma cells compared with siControl cells treated with asoprisnil (Fig. 4E).

Effects of GADD153 RNA interference on asoprisnil-induced increase in the protein contents of GADD153-target molecules, GADD34 and TRB3, in cultured leiomyoma cells. GADD153 is known to target proapoptotic molecules such as GADD34 (21) and TRB3 (29). To examine the association of GADD153 with GADD34 and TRB3 induction in asoprisnil-induced apoptosis in cultured leiomyoma cells, we used the siRNA technique to knockdown GADD153 gene. RNA interference of GADD153 resulted in the decrease in GADD34 and TRB3 protein contents in cultured leiomyoma cells (Fig. 5, A and B). Asoprisnil-induced increase in GADD34 and TRB3 protein contents were significantly \( (P < 0.01) \) decreased in siGADD153 cells compared with siControl cells (Fig. 5, A and B).

Effects of GADD153 RNA interference on asoprisnil-induced decrease in Bcl-2 and increase in Bax and Bak protein contents in cultured leiomyoma cells. RNA interference of GADD153 caused an increase in Bcl-2 protein content (Fig. 6A) and a decrease in protein contents of Bax and Bak in cultured leiomyoma cells (Fig. 6, B and C). RNA interference
of GADD153 resulted in an inhibition of asoprisnil-induced reduction in Bcl-2 protein content in the cells (Fig. 6A). However, asoprisnil-induced increase in Bax and Bak protein contents was significantly (*P < 0.01) decreased in siGADD153 cells compared with siControl cells (Fig. 6, B and C).

**DISCUSSION**

We have previously shown that the SPRM asoprisnil induces apoptosis in cultured leiomyoma cells in a cell-type specific manner by acting through intrinsic and extrinsic apoptotic pathways without affecting the cell proliferation and apoptosis of normal myometrial cells (5, 36). In this study, we further elucidated the mechanism by which asoprisnil induces apoptosis of cultured leiomyoma cells. The present study provided novel evidence that asoprisnil elicits ER stress-induced apoptosis in cultured leiomyoma cells and that the proapoptotic transcription factor GADD153 plays a vital role in asoprisnil-induced apoptosis of cultured leiomyoma cells through the modulation of apoptosis-related molecules such as GADD34, TRB3, and the Bcl-2 family of proteins.

In the present study, the time-course and dose-dependency experiments revealed that asoprisnil treatment rapidly triggered the induction of ubiquitin protein content, the UPR signaling markers such as ER stress sensors p-PERK, p-eIF2α, ATF4, and ER chaperone proteins GRP78 and GRP94, and cleaved PARP protein content in cultured leiomyoma cells. Furthermore, the dose-dependency experiment confirmed that the effects of asoprisnil on the UPR signaling mediated by the PERK-eIF2α-ATF4 branch in cultured leiomyoma cells were similar to those of the ER stress inducer tunicamycin, except that asoprisnil treatment did not activate the UPR branch mediated by IRE1α and XBP-1. However, the reason for the unresponsiveness of the IRE1α and XBP-1 branch of the UPR in cultured leiomyoma cells exposed to asoprisnil treatment remains unknown.

Both asoprisnil and tunicamycin augmented ubiquitin protein content in cultured leiomyoma cells. The time-course study revealed that ubiquitin protein content started to increase at 2 h in cultured leiomyoma cells treated with 10^{-7} M asoprisnil before the induction of the UPR markers. Because one of the triggers of ER stress is the accumulation of misfolded proteins, as occurs with the ER stress inducer tunicamycin, it is speculated that asoprisnil may act to trigger the UPR through the accumulation of ubiquitinated proteins. These results suggest that the UPR signaling may act to promote cell survival of cultured leiomyoma cells in response to the accumulation of proteins caused by asoprisnil treatment because an activation of the PERK-eIF2α signaling pathway attenuates protein translation and accumulation (14, 19, 34), and because GRP78 protects cells from apoptosis (35). However, the precise mechanism by which asoprisnil increases ubiquitin protein contents in cultured leiomyoma cells remains unknown. Further study is required to elucidate whether asoprisnil treatment impairs proteasomal degradation of ER-associated unfolded or misfolded proteins in cultured leiomyoma cells.

By contrast, a proapoptotic transcription factor, GADD153 protein, markedly increased in cultured leiomyoma cells treated with asoprisnil starting at 6 h, indicating the shift from the cytoprotective UPR to an initiation of GADD153-regulated apoptotic machinery. Several investigators have demonstrated...
that GADD153 is implicated in ER stress-induced apoptosis (21, 32, 50) and that activation of PERK, ATF6, and IRE1 induces the transcription of GADD153 (12, 15, 41, 48). Although the precise mechanism by which GADD153 induces apoptosis remains poorly understood, GADD153 is shown to target apoptosis-related molecules such as Bcl-2 (24), GADD34 (21), and TRB3 (29). In view of the induction of GADD153 in cultured leiomyoma cells treated with asoprisnil, we examined the association of GADD153 with these GADD153-targeted molecules and another proapoptotic Bcl-2 family of proteins, Bax and Bak.

In the present study, RNA interference of GADD153 reduced the protein content of a key death substance, PARP, that represents a hallmark for the apoptotic paradigm (33) and the
rate of TUNEL-positive cultured leiomyoma cells that indicates DNA rupture in cultured leiomyoma cells in the presence or absence of asoprisnil. These results suggest that GADD153 plays a role, at least in part, in asoprisnil-induced apoptosis of cultured leiomyoma cells. In the next series of knockdown experiments, we examined whether GADD153 modulates the induction of GADD34 and TRB3 protein contents in cultured leiomyoma cells in response to asoprisnil treatment. RNA interference of GADD153 resulted in a significant decrease in asoprisnil-induced GADD34 and TRB3 protein contents compared with siControl cells treated with asoprisnil. GADD34 is a DNA damage-inducible factor that enhances apoptosis (1). During ER stress, GADD153 and ATF4 transactivate GADD34 (20), which in turn forms a complex with protein phosphatase 1 that specifically promotes the dephosphorylation of eIF2α, thereby promoting recovery from translational inhibition of proteins (25). An inhibition of eIF2α dephosphorylation is shown to protect cells from ER stress-induced apoptosis (4). On the basis of these findings, it is speculated that GADD34 may act to promote asoprisnil-induced apoptosis of cultured leiomyoma cells by augmenting unfolded and/or misfolded protein synthesis.

Furthermore, RNA interference of GADD153 suppressed asoprisnil-induced TRB3 protein content in cultured leiomyoma cells. TRB3 is a mammalian homolog of Drosophila tribbles and is upregulated during ER stress inducer thapsigargin-induced apoptosis (30). ATF4 and GADD153 cooperate to
activate the promoter activity of TRB3 (29, 31). Ohoka et al. (29) demonstrated that knockdown of ATF4 or GADD153 repressed tunicamycin-induced TRB3 induction and that knockdown of TRB3 decreased ER-stress-induced apoptosis, suggesting the involvement of TRB3 in GADD153-dependent cell death. A recent study has demonstrated that TRB3 blocks activation of a serine-threonine kinase Akt (10). Because the Akt pathways are activated during thapsigargin- or tunicamycin-induced ER stress and counteract ER stress-induced cell death (16), TRB3 is thought to promote ER stress-induced apoptosis by interfering with Akt activation. Thus it seems reasonable to speculate that an increased level of TRB3 caused by GADD153 may play a role in asoprisnil-induced apoptosis of cultured leiomyoma cells. However, it remains to be elucidated whether asoprisnil affects the activation of the Akt pathway in these cells.

Accumulating evidence suggests that the Bcl-2 family members regulate both mitochondrial and ER stress-induced apoptotic pathways. Bcl-2 targeted to the ER is shown to block tunicamycin-induced mitochondrial cytochrome c release and caspase-3 activation (13) and protect Bax-induced apoptosis (42). Bax and Bak double deficient murine embryonic fibroblasts are resistant to thapsigargin- and tunicamycin-induced caspase-3 activation (13) and protect Bax-induced apoptosis of cultured leiomyoma cells. However, it remains to be elucidated whether asoprisnil affects the activation of the Akt pathway in these cells.

In our study, knockdown of GADD153 suppressed asoprisnil-induced decrease in Bax and Bak protein contents and attenuated asoprisnil-induced decrease in Bcl-2 protein content in cultured leiomyoma cells, suggesting that GADD153 mediates not only asoprisnil-induced Bcl-2 downregulation but also asoprisnil-induced Bax and Bak upregulation. Our results are in agreement with reports showing that GADD153 represses the promoter of Bcl-2 (24) and upregulates Bax expression in celecoxib-induced apoptosis of cervical cancer cells (18). It seems likely that the decreased level of Bcl-2 protein may stimulate the activation of Bax and Bak, leading to the promotion of ER stress-induced and mitochondrial apoptosis in cultured leiomyoma cells exposed to asoprisnil treatment. However, it remains to be elucidated whether GADD153 directly stimulates the promoters of Bax and Bak. Further study will be necessary to elucidate whether GADD153 targets Bax and Bak during the ER stress.

Additionally, several authors have demonstrated that tunicamycin upregulates death receptor 5 (DR5), which is an apoptosis-inducing membrane receptor for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (39) and that GADD153 enhances DR5 expression in cancer cells (47). These results indicate the link between ER stress-induced apoptosis and TRAIL-mediated apoptosis. We have recently shown that asoprisnil elicits TRAIL-induced apoptosis in cultured leiomyoma cells (36). This fact suggests that GADD153 may be involved in the asoprisnil-induced TRAIL-mediated apoptosis in cultured leiomyoma cells through induction of DR5 expression.

In conclusion, we demonstrated, for the first time, that the SPRM asoprisnil activates the UPR signaling pathway and subsequently elicits ER stress-induced apoptosis in cultured leiomyoma cells. The results of the knockdown study suggest that GADD153 may play a vital role in promoting asoprisnil-induced apoptotic machinery in cultured leiomyoma cells through upregulating GADD34, TRB3, Bax, and Bak and by downregulating Bcl-2 protein levels. Our results provide a novel insight into the molecular mechanism underlying the growth-inhibitory action of asoprisnil on uterine leiomyomas.

REFERENCES


DISCLOSURES

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